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SHORT COMMUNICATION

Sulfinpyrazone *C*-glucuronidation is catalyzed selectively by human UDP-glucuronosyltransferase 1A9 (UGT1A9)

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Running title: Characterization of sulfinpyrazone C-glucuronidation

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Non-standard abbreviations:

HEK293, human embryo kidney 293 cells; HLM, human liver microsomes; HPLC, high performance liquid chromatography; SFZ, sulfinpyrazone; SFZG, sulfinpyrazone β -D-glucuronide; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

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ABSTRACT

The uricosuric agent sulfinpyrazone (SFZ) is metabolized via *C*-glucuronidation, an uncommon metabolic pathway, in humans. The present study aimed to characterize SFZ glucuronidation by human liver microsomes (HLM) and identify the hepatic forms of UDP-glucuronosyltransferase responsible for this pathway. Incubations of SFZ with HLM formed a single glucuronide that was resistant to β -glucuronidase and acid hydrolysis, consistent with formation of a *C*-glucuronide. Mass spectral analysis confirmed the identity of the metabolite as SFZ glucuronide (SFZG). SFZ *C*-glucuronidation by HLM exhibited Michaelis-Menten kinetics, with mean (\pm SD) K_m and V_{max} values of $51 \pm 21 \mu\text{M}$ and $2.6 \pm 0.6 \text{ pmol/min} \cdot \text{mg}$, respectively. Fifteen recombinant human UGTs, expressed in HEK293 cells, were screened for their capacity to catalyze SFZ *C*-glucuronidation. Of the hepatically expressed enzymes, only UGT1A9 formed SFZG. UGT 1A7 and 1A10, which are expressed in the gastrointestinal tract, also metabolized SFZ but rates of metabolism were low compared to UGT1A9. SFZ glucuronidation by UGT1A9 exhibited 'weak' negative cooperative kinetics, which was modeled by the Hill equation ($S_{50} 16 \mu\text{M}$). The data indicate that UGT1A9 is the enzyme responsible for hepatic SFZ *C*-glucuronidation, and that SFZ may be used as a substrate 'probe' for UGT1A9 activity in HLM.

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Sulfinpyrazone (1,2-diphenyl-4-(phenylsulfinylethyl)-3,5-pyrazolidinedione; SFZ) (Fig. 1) is a uricosuric agent used primarily to treat hyperuricemia associated with gout (Pittman and Bross, 1999). SFZ also possesses antiplatelet and antithrombotic actions (Margulies et al., 1980). It has been demonstrated that *C*-glucuronidation is a major biotransformation pathway of SFZ in humans (Dieterle et al., 1975 and 1980), resulting in the formation of a conjugate (SFZG) in which the pyrazolidine ring is attached directly to glucuronic acid via a C-C bond (Fig. 1).

Glucuronidation reactions are catalyzed by the enzyme UDP-glucuronosyltransferase (UGT) and involve the covalent linkage of glucuronic acid, derived from the co-factor UDP-glucuronic acid (UDPGA), to a nucleophilic atom on the substrate. UGT exists as a superfamily of enzymes; nineteen human UGT proteins that utilize UDPGA as cofactor have been identified to date, and these have been classified into two families (UGT1 and UGT2) based on sequence identity (Mackenzie et al., 2005). The individual UGTs exhibit distinct, but overlapping, substrate and inhibitor selectivities and differ in terms of regulation of expression, genetic polymorphism (occurrence and frequency), and other factors known to influence the activity of drug metabolizing enzymes in humans (Miners and Mackenzie 1991; Miners et al., 2004). Despite recognition of the importance of glucuronidation as a clearance and detoxification mechanism for drugs, environmental chemicals and endogenous compounds, relatively few selective substrates and inhibitors useful for the reaction phenotyping of glucuronidated compounds have been identified to date (Court, 2005; Miners et al., 2006).

The majority of glucuronidation reactions in humans involve the formation of *O*- and *N*-glucuronides via conjugation of alcohols, carboxylic acids and amines (Miners and

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Mackenzie 1991). In contrast, *C*-glucuronidation is rare. SFZ, phenylbutazone, and feprazone, all derivatives of 1,2-diphenyl-3,5-pyrazolidinedione, provide examples of compounds known to form *C*-glucuronides in humans (Richter et al., 1975; Yamaguchi et al., 1979). *C*-Glucuronidation occurs via the acidic carbon atom of the pyrazolidinedione ring, which is alpha to two electron-withdrawing carbonyl groups. Given the unique nature of *C*-glucuronidation reactions, we reasoned that the glucuronidation of SFZ (and probably related compounds) might be catalyzed selectively by a single human UGT, or by a limited number of UGT enzymes. Furthermore, although SFZ has been in clinical use for several decades, the kinetics of SFZ *C*-glucuronidation by human liver has not been investigated. This study aimed to characterize the kinetics of SFZ *C*-glucuronidation by human liver microsomes and identify the human UGT enzyme(s) responsible for this pathway.

MATERIALS AND METHODS

Materials

Alamethicin (from *Trichoderma viride*), β -glucuronidase (from *Escherichia coli*), SFZ, and UDP-glucuronic acid (UDPGA; trisodium salt) were purchased from Sigma-Aldrich (Sydney, Australia). All other reagents and solvents were of analytical reagent grade.

Methods

Human liver microsomes and recombinant UGT proteins: Human livers (HL10, 12, 13, and 40) were obtained from the human liver 'bank' of the Department of Clinical Pharmacology, Flinders Medical Centre. Microsomes were prepared by differential centrifugation, as described by Bowalgaha et al. (2005) and stored at -80°C until use. Human liver microsomes (HLM) were activated by pre-incubation with alamethicin (50 μ g/mg protein) on ice for 30 min prior to use in incubations (Boase and Miners, 2002). UGT 1A1, 1A3, 1A4, 1A5, 1A6,

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1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17, and 2B28 cDNAs were stably expressed in a human embryonic kidney cell line (HEK293), as described previously (Stone et al., 2003; Uchaipichat et al., 2004). Expression of each UGT protein was demonstrated by immunoblotting with a commercial UGT1A antibody and a nonselective UGT antibody (raised against purified mouse Ugt) (Uchaipichat et al., 2004). Activities of recombinant UGTs (except UGT 1A4, 1A5, and 2B10) were confirmed using the nonselective substrate 4-methylumbelliferone, as described by Rowland et al. (2006). The activity of UGT1A4 was confirmed using trifluoperazine as substrate (Uchaipichat et al., 2006).

SFZ glucuronidation assay: Incubation mixtures, in a total volume of 200 μ l, contained HLM or HEK293 cell lysate protein (2 mg/ml), UDPGA (5 mM), $MgCl_2$ (4 mM), and SFZ (11-12 substrate concentrations in the range of 2.5-400 μ M for kinetic studies) in 0.1 M phosphate buffer, pH 7.4. SFZ stock solutions were prepared in methanol, such that incubations contained 1% solvent (v/v). This concentration of methanol has been shown to have a negligible effect on UGT activities (Uchaipichat et al., 2004). After a 5-min preincubation at 37°C, reactions were initiated by the addition of UDPGA. Incubations were carried out for 3 h at 37°C in a shaking water bath. 'Blank' incubations, which contained methanol (1% v/v), were performed in the absence of UDPGA. Reactions were terminated by the addition of an ice-cold 4% acetic acid/96% methanol (200 μ l). Following centrifugation (12,000g for 10 min), a 40- μ l aliquot of the supernatant fraction was injected onto an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) fitted with a Waters Nova-Pak C18 column (150 \times 3.9 mm, 4 μ m; Milford, MA) with a Phenomenex SecurityGuard cartridge (Phenomenex, Torrance, CA). Peaks were separated with 90% 10 mM triethylamine, pH 2.5 (adjusted with $HClO_4$)/10% acetonitrile (mobile phase A) and 100% acetonitrile (mobile phase B) as follows: initial conditions of 80%A/20%B for 2 min, followed by a linear

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gradient over 10 min to 60%A/40%B, which was held for 1 min before returning to the starting conditions. The mobile phase flow rate was 1.0 ml/min and peaks were monitored by UV detection at 240 nm (the absorbance maximum for SFZ in the mobile phase). Under these conditions, retention times for SFZG and SFZ were 6.0 and 12.1 min, respectively. SFZG was quantified by comparison of peak areas to those of an SFZ external standard curve prepared over the concentration range 0.25-2 μ M. SFZ standard curves were linear over this concentration range, with r^2 values > 0.99. The lower limit of quantification, defined as 5-times background, was 0.01 μ M (equivalent to a rate of product formation of 0.03 pmol/min \cdot mg). Overall within-day assay reproducibility was assessed by measuring SFZG formation in 10 separate incubations of the same batch of HLM. Within-day coefficients of variation were 1.8% and 2.7% for substrate concentrations of 10 and 250 μ M, respectively. The formation of SFZG was linear with incubation times to at least 4 h and microsomal protein concentrations to at least 4 mg/ml (data not shown).

Isolation of SFZG: SFZ was incubated with pooled HLM (10 mg), in a total volume of 5 ml, for 15 h. The reaction was terminated with 85% H_3PO_4 (50 μ l) and centrifuged (12,000g for 10 min). The supernatant fraction was separated and applied to a C18 Sep-Pak solid-phase extraction cartridge (Waters, Milford, MA), which was preconditioned with 2 ml of methanol, 5 ml of water, and 2 ml of acidified water (pH 2). Following application of the sample, the cartridge was washed with 1 ml of acidified water (pH 2) and 5 ml of water, dried under vacuum, and eluted with methanol (4 ml). The eluate was evaporated to dryness under nitrogen and the residue was reconstituted in 200 μ l of methanol/acetic acid/water (50:2:48, v/v). The fraction corresponding to the SFZG peak was separated by HPLC (see above), again extracted on a Sep-Pak C18 cartridge, and eluted with methanol for mass spectral

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analysis. A portion of this eluate was evaporated to dryness under nitrogen and reconstituted in water for the acid hydrolysis study (see below).

Identification of SFZG: The structural identity of the SFZG peak formed by incubations of HLM was confirmed by mass spectrometry and by enzymatic and acid hydrolysis. Mass spectra were acquired using a Micromass Quattro micro tandem quadrupole mass spectrometer (Waters, Manchester, UK) with electrospray ionization (ESI) in positive ion mode. The sample was introduced into the ESI source via an integrated syringe pump at a flow rate of 10 $\mu\text{l}/\text{min}$. Instrument parameters were: capillary voltage, 3.2 kV; cone voltage, 35 V; source temperature, 90°C; desolvation temperature, 300°C; desolvation gas flow, 250 l/h; collision energy, 20 V; and gas cell Pirani pressure 3.78×10^{-3} mbar.

For the enzymatic hydrolysis experiment, an SFZ glucuronidation incubation sample, in a total volume of 200 μl , was terminated with 85% H_3PO_4 (2 μl) and centrifuged (12,000g for 10 min). The supernatant fraction was decanted and adjusted to pH 7 with 6 M KOH.

Enzymatic hydrolysis was performed by incubation with β -glucuronidase (100 units/ml) at 37°C for 2 h. Control incubations without β -glucuronidase were performed simultaneously.

Acid hydrolysis was performed by adding 100 μl of 6 M HCl to 200 μl of the purified aqueous solution of SFZG (see above) and heating at 90°C for 1 h (since acid hydrolysis of incubation mixtures gave an interfering peak close to that of SFZG). Control incubations without HCl were performed simultaneously.

Data analysis: Kinetic constants for SFZ C-glucuronidation by HLM and recombinant UGTs were obtained by fitting experimental data to the Michaelis-Menten or Hill equations using EnzFitter (Biosoft, Cambridge, UK), to obtain estimates of K_m (or S_{50}), maximal velocity

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(V_{max}), and the Hill coefficient, n (as described by Uchaipchat et al., 2004). Goodness of fit to kinetic models was assessed by comparison of the F -statistic, coefficient of determination (r^2), parameter standard errors, and 95% confidence intervals.

RESULTS AND DISCUSSION

HPLC analysis of incubations containing SFZ and UDPGA revealed a metabolite peak with a retention time of 6.0 min, which was not observed in incubations performed without UDPGA or SFZ. The putative SFZG was resistant to β -glucuronidase and acid hydrolysis, as reported previously for *C*-glucuronides (Richer et al., 1975; Dieterle et al., 1975; Yasuda et al., 1982). The identity of the SFZG peak was thus confirmed by electrospray mass spectrometry in positive ion mode. Mass spectral analysis showed the presence of a protonated molecular ion $[M+H]^+$ at m/z 581, which is consistent with the molecular weight of protonated SFZG. The major fragment ions, detected at m/z 455, 405, and 279, correspond to the loss of phenylsulfoxide, glucuronic acid, and both groups, respectively. Although phenylbutazone is reported to form both *C*- and *O*-glucuronides (the latter presumably via an enol), only SFZ *C*-glucuronide has been observed in the urine of humans administered SFZ (Dieterle et al., 1975 and 1980). The occurrence of a single SFZG peak in microsomal incubations, which was unaffected by β -glucuronidase and acid hydrolysis, is consistent with the *in vivo* observations. The resistance of *C*-glucuronides to β -glucuronidase and acid hydrolysis should be considered when screening compounds containing an acidic carbon for glucuronide formation.

Microsomal SFZ *C*-glucuronidation exhibited Michaelis-Menten kinetics in the four livers investigated (Fig. 2A). Mean (\pm SD) derived K_m and V_{max} values for the four livers were 51 ± 21 μ M and 2.6 ± 0.6 pmol/min \cdot mg, respectively. As noted previously, standard curves were

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prepared using SFZ. Thus, rates of SFZ C-glucuronide formation and V_{max} values for this metabolite should be considered 'apparent'.

UGT 1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17, and 2B28 were screened for SFZ C-glucuronidation activity at substrate concentrations of 10, 50, and 250 μM . Of these enzymes, only UGT 1A7, 1A9, and 1A10 converted SFZ to its C-glucuronide. UGT1A9 exhibited the highest activity whereas UGT1A7 and 1A10 exhibited lesser activity (<14% that of UGT1A9) (Fig. 2C). Given the lower activities of UGT 1A7 and 1A10, and lack of expression of these enzymes in liver (see below), kinetic studies were performed only with UGT1A9. Although kinetic data were adequately fitted to the Michaelis-Menten equation, SFZ C-glucuronidation by UGT1A9 was best described by the Hill equation (with $n = 0.77 \pm 0.01$, i.e., negative cooperativity) (Fig. 2B); all goodness of fit parameters (see *Data Analysis*) were superior using this model. The S_{50} and V_{max} (parameter \pm SE of parameter fit) values were $16 \pm 0.3 \mu\text{M}$ and $3.2 \pm 0.02 \text{ pmol/min} \cdot \text{mg}$, respectively.

UGT1A9 is expressed in liver and a range of other human tissues (Tukey and Strassburg 2000). In contrast, UGT 1A7 and 1A10 are expressed only in the gastrointestinal tract and hence cannot contribute to SFZ C-glucuronidation by HLM (or hepatic SFZ clearance in vivo). Thus, on the basis of the screening data, it is postulated that human hepatic SFZ C-glucuronidation is catalyzed solely by UGT1A9 and SFZ may thus serve as a substrate 'probe' for UGT1A9 activity in HLM. It should be noted that no selective inhibitors of UGT1A9 are currently available, and hence confirmatory inhibition experiments are not possible. Whereas SFZ is a selective substrate of UGT1A9 we have demonstrated recently that both SFZ and phenylbutazone inhibit most UGT1A subfamily enzymes (Uchaipichat et al., 2006), although the lowest IC_{50} values were associated with those enzymes shown here to glucuronidate SFZ (UGT 1A7, 1A9 and 1A10). This observation provides further evidence

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for the principle that substrate and inhibitor selectivities may not be identical with drug metabolizing enzymes. Interestingly, differences in the kinetic models for SFZ C-glucuronidation by HLM (Michaelis Menten) and UGT1A9 expressed in HEK293 cells ('weak' negative cooperativity) were observed here. The reasons for this are unclear, but non-Michaelis Menten kinetics are not uncommonly observed for xenobiotic glucuronidation reactions by recombinant UGTs (Miners et al., 2004 and 2006), including UGT1A9 (Uchaipichat et al., 2004; Bowalgaha et al., 2005).

Apart from SFZ, propofol has been proposed as a selective substrate for hepatic UGT1A9 activity (Court, 2005), but detailed comparative kinetic studies have not been published. Like SFZ, propofol also appears to be a substrate for UGTs expressed exclusively in the gastrointestinal tract (viz. 1A7, 1A8 and 1A10) (Court, 2005). Although the apparent V_{max} for SFZ C-glucuronidation by HLM is relatively low, SFZG formation during the course of a standard incubation at a substrate concentration corresponding to the K_m is almost 500 pmol, which is very easily measurable. When considered as a substrate probe for human liver microsomal UGT1A9 activity SFZ offers advantages compared to propofol, including; well characterized hyperbolic kinetics, ease of handling (solid vs. liquid), solubility, and negligible non-specific binding to HLM (< 5% for SFZ (O. Kerdpin and J.O. Miners, unpublished results) vs. 95% for propofol (Soars et al., 2002)).

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FOOTNOTE

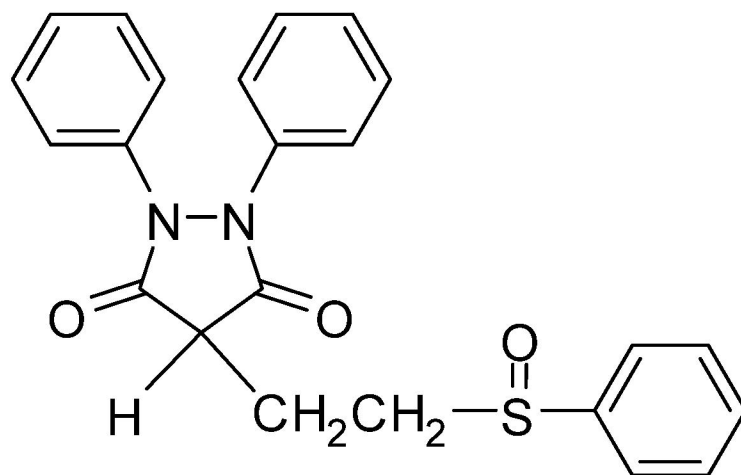
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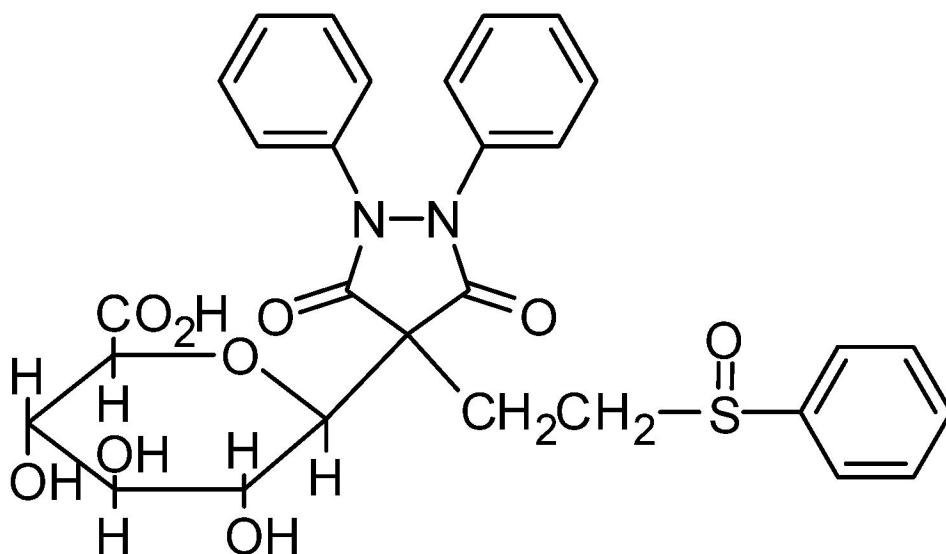
LEGENDS FOR FIGURES

Fig. 1. Structures of sulfinpyrazone and sulfinpyrazone *C*-glucuronide.

Fig. 2. Representative Eadie-Hofstee plots for sulfinpyrazone *C*-glucuronide by human liver microsomes (liver 12) (panel A) and UGT1A9 (panel B), and rates of sulfinpyrazone *C*-glucuronidation by recombinant UGTs (panel C). Points are experimentally determined values (means of duplicate measurements at each concentration), while the solid lines are the computer-generated curves of best fit.



Sulfinpyrazone



Sulfinpyrazone C-glucuronide

Fig. 1.

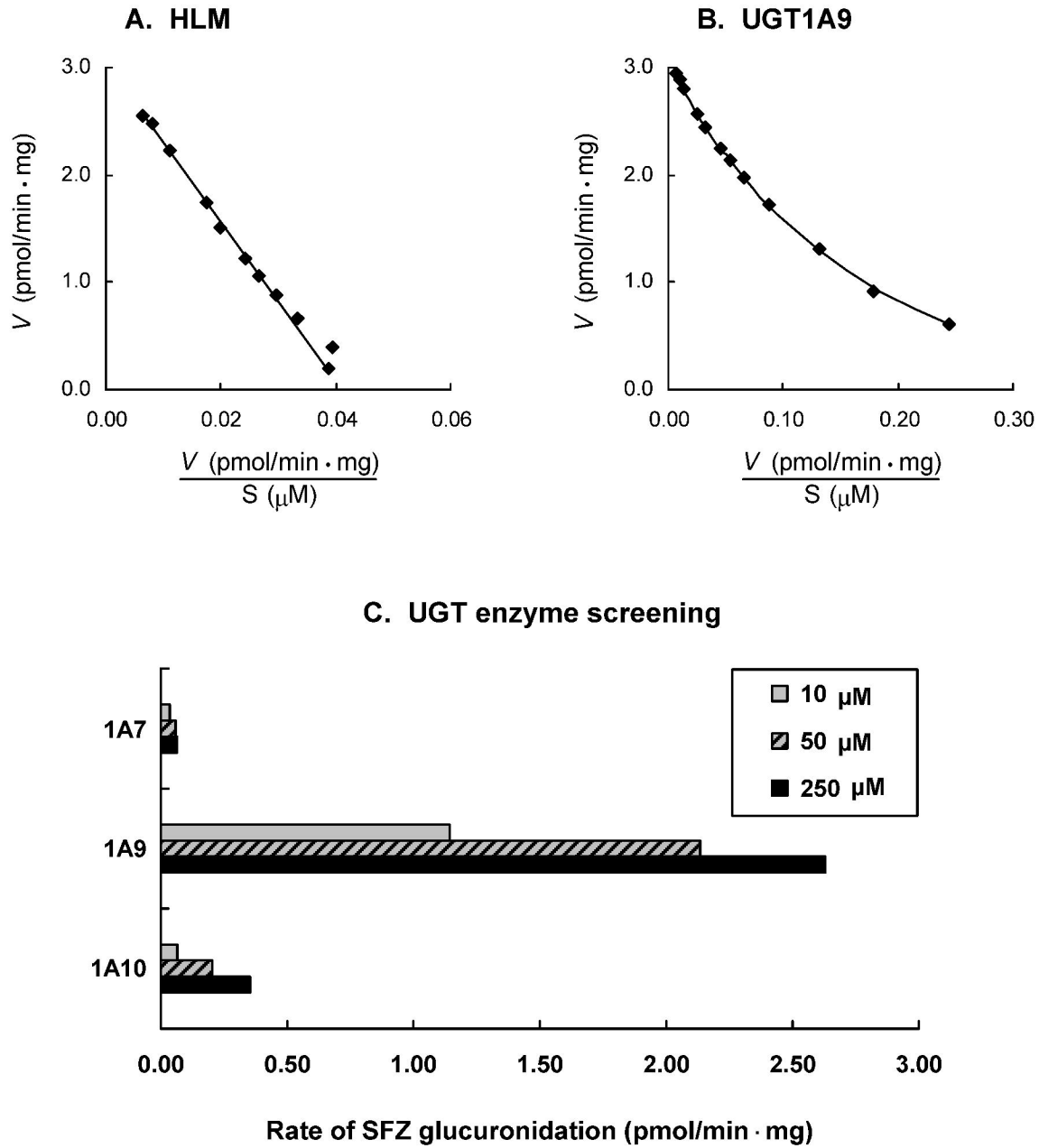


Fig. 2.